C1

variation of this sequence includes ATG GGA CAT ACG ATG (SEQ ID NO:311), both of which sequences encode the amino acid sequence MGHTM, which corresponds to amino acids 1-5 of SEQ ID NO:48.

2. Delete the paragraph beginning at page 81, line 4, and substitute therefor the following paragraph:

C2

In a further example, if four conservative substitutions were localized in the region corresponding to amino acids 69-94 of SEQ ID NO:48, examples of conservatively substituted variations of this region, QKDSK MVLAI LPGKV QVWPE YKNRTI, would include:

NKDSK MVVAI LPGKV QVFPE YKNKTI (SEQ ID NO:294) and

QKDAK MVLAI LPGRV QMWPE YKQRTI (SEQ ID NO:295) and the like, where conservative substitutions listed in Table 2 (in the above example, conservative substitutions are underlined).

3. Delete the paragraph beginning at page 198, line 18, and substitute therefor the following paragraph:

C3

The FLAG sequence (DYKDDDDK (SEQ ID NO:296)) was inserted at the junction separating the sequence encoding the signal peptide and the sequence encoding the mature polypeptide (e.g., mature coding region) for each of the human B7-1 and CD28BP clones (e.g., CD28BP-15) using the ExSite PCR site-directed mutagenesis kit (Stratagene, San Diego, CA) according to manufacturer's instructions. The nucleotide sequences corresponding to the signal sequence and mature coding region were determined for each shuffled nucleotide sequence by comparison with the known sequences corresponding to the signal sequence and mature coding region for hB7-1. Mutagenesis primers were designed with the FLAG sequence flanked by 24 nucleotides of signal and mature coding sequence specific to each clone. Plasmid DNA was prepared and purified from the cDNA libraries following standard procedure in Maniatis et al., Molecular Cloning: A laboratory Manual, Cold Spring Harbor, NY (1987).

4. Delete the paragraph beginning at page 216, line 23, and ending at page 217, line 23, and substitute therefor the following paragraph:

Sequence analysis of CD28BP-15 indicated the amino acid sequence comprised a chimera derived principally from human, bovine, and rabbit sequences (Fig. 8B). The remaining clones selected from the libraries resulting from second round of recombination that displayed preferential binding to CD28 over CTLA-4 shared about 74 to about 99% sequence identity with CD28BP-15 based on sequence alignment comparisons (using DNASTAR or Vector NTI algorithm with default parameters as described above), illustrating the diversity of clones having the preferential binding properties. The nucleotide and amino acid sequence identities of CD28BP-15 with human B7-1 were 73% and 61%, respectively (using DNASTAR or Vector NTI algorithm with default parameters). Based on amino acid sequence alignments, all of the selected CD28BP clones exhibiting preferential binding to CD28 over CTLA-4 relative to hB7-1 contained a substitution of valine for isoleucine at amino acid position 49 (Ile49Val) of the mature CD28BP-15 amino acid sequence (corresponding to alignment with the mature hB7-1 sequence). This substitution corresponds to a substitution at amino acid position 85 in the full-length CD28BP-15 and amino acid position 83 in full-length human B7-1, since CD28BP-15 includes two additional amino acid residues in the putative sequence. Although Ile49 does not appear to be directly involved in the interaction of B7-1 with CTLA-4 (see, e.g., Stamper, C. et al. (2001) *Nature* 410:608-611), substitution Ile49Ala was previously shown to completely abolish binding of B7-1 to both CD28 and CTLA-4, suggesting the importance of this residue in the ligand binding (Peach, R.J. et al. (1995) *J Biol Chem* 270:21181-7). Although the Ile49Val substitution is also considered a conservative replacement, our data suggest that mutations derived from naturally existing genes, in this case from the bovine B7-1 gene sequence, provide improved means to search for altered functional properties in proteins. Notably, bovine CD28 receptor is the only exception among CD28 and CTLA-4 receptor amino acid sequences analyzed from 12 different species in which the hexapeptide MYPPPY (SEQ ID NO:297) and Gly-66 are not fully conserved in the receptor sequence (i.e., MYPPPY is replaced by LYPPPY (SEQ ID NO:312), and Gly-66 is replaced by valine (see Metzler, W.J. et al. (1997) Nat Struct Biol 4:527-31). The hexapeptide MYPPPY is conserved in the F-G loop of both CD28 and CTLA-4 in a variety of mammalian species. Mutation of any residue in the MYPPPY sequence

C4 cont

leads to reduced binding to B7-1 and B7-2 (see id.), and all these residues, with the exception of Pro101, are in direct contact with B7-1 (Stamper, C. et al. (2001) Nature 410:608-611), suggesting that changes in this region of bovine CD28 receptor have driven the natural evolution of bovine B7-1 to acquire properties that also benefited the in vitro evolution of CD28BP described herein.

5. Delete the paragraph beginning at page 226, line 2, and ending at page 227, line 3, and substitute therefor the following paragraph:

C5

Table 5 shows for positions of nucleotide residues and corresponding amino acid residues of the signal peptide sequence and representative ECD domains of selected CD28BP and CTLA-4BP clones, and equivalent positions in WT hB7-1 ECD, and the last three amino acid residues at the 3' end of each ECD of a selected NCSM clone or WT hB7-1. The present invention provides for ECD domains of the NCSM polypeptides (and nucleic acid sequences encoding such polypeptides) that lack the signal peptide sequence, such that the first about 33 or about 34 amino acids (or about 99 or about 102 nucleic acids encoding same, respectively) of each NCSM ECD polypeptide (or nucleic acid encoding said polypeptide) are absent. For example, in CD28BP-15 polypeptide (SEQ ID NO:66), the amino acid sequence of the signal peptide comprises MGHTMKWGSLPPKRPCLWLSQLLVLTGLFYFCSG (SEQ ID NO:315) (see Fig. 2A), and the nucleic acid sequence that encodes the signal peptide comprises at least about the first 102 nucleotide residues of SEQ ID NO:19. The signal peptide sequence of NCSM molecules may vary in length. One of ordinary skill in the art can readily determine the amino acid sequence of an NCSM molecule that comprises the signal peptide sequence by aligning the full-length amino acid sequence of WT hB7-1 with the full-length amino acid sequence of the NCSM molecule, comparing the signal peptide sequence of WT hB7-1 with the corresponding sequence of the NCSM molecule, and determining the segment of the full-length amino acid sequence of the NCSM molecule that corresponds to the signal peptide. The nucleic acid sequence that encodes the signal peptide of hB7-1 comprises the nucleotide residues of SEQ ID NO:273 that encode the signal peptide of SEQ ID NO:278. The nucleic acid sequence that encodes the signal peptide of an NCSM molecule can be similarly determined by comparison of

C5 cont

the full-length nucleic acid sequence of WT hB7-1 with the full-length nucleic acid sequence of the NCSM molecule.

6. Delete the paragraph beginning at page 227, line 4, and substitute therefor the following paragraph:

C6

Figure 14A shows a schematic representation of a hB7-1-ECD fused to an E-epitope amino acid sequence and a hexa-His tag (SEQ ID NO:299) amino acid. The amino acid sequences corresponding to the E-epitope and hexa-His tag, and selected amino acids of the ECD, are shown.

7. Delete the paragraph beginning at page 230, line 7, and substitute therefor the following paragraph:

C7

Figure 14B shows a representation of a soluble WT human B7-1-ECD-Ig sequence, including the signal domain, ECD, Factor Xa (IEGR (SEQ ID NO:300)), VT or GVT linker, and human B7 hinge CH2-CH3 domain of the Fc region of IgG1 corresponding to the sequence shown at GenBank Accession No. P01857. The amino acid residues positioned at the beginning and end of a representative ECD domain and 5' end of the human B7 hinge CH2-CH3 domain are shown. A NCSM-ECD-Ig sequence would be comparable to that shown for hB7-1ECD-Ig in Figure 14B.

8. Delete the paragraph beginning at page 231, line 10, and substitute therefor the following paragraph:

C8

In another aspect, a non-dimerizing Ig-Fc domain (PKSCDKTHTCPPCP (SEQ ID NO:298) → PKSSDKTHTSPPSP (SEQ ID NO:301)) was engineered by PCR mutagenesis (Stratagene, La Jolla, CA) to mutate the cysteine residues within the Ab hinge region to serine residues so as to prevent the formation of NCSM-ECD-Ig or hB7-1-ECD-Ig homodimers covalently linked by disulfide bonds between the hinge-CH2 cysteines of neighboring NCSM-ECD-Ig or hB7-1-ECD-Ig molecules. This non-dimerizing Ig-Fc domain can alternatively be used as the Ig portion in an NCSM-ECD-Ig or hB7-1-ECD-Ig fusion protein prepared as

described above. Affinity purified huB7-1-ECD-IgδCys, comprising hB7-1-ECD fused to Ig in which the cysteines were mutated (represented by delta or δCys) was shown to have a molecular weight of ~70 kDa (molecular size of non-disulfide linked Fc fusion monomer) (Figure 16). The present invention provides similarly prepared Cys-mutant Ig fusion proteins, NCSM-ECD-IgδCys or NCSM-trunECD-IgδCys, and nucleic acid sequences encoding such proteins.

9. Delete the paragraph beginning at page 237, line 30, and ending at page 238, line 14, and substitute therefor the following paragraph:

Multimeric NCSM molecules are generated using leucine zippers. In this approach, leucine repeats are utilized to generate multimeric NCSM molecules that have properties which are equivalent or substantially identical to properties of the crosslinked NCSM multimers described above generated by crosslinking using goat anti-human IgG Fc mAbs (and crosslinked NCSM fragments having such properties). The use of leucine zippers to oligomerize proteins is well known (see, e.g., Rieker and Hu (2000) Methods Enzymol. 323:282-96; Behncken et al. (2000) J. Biol. Chem. 275:17000-17007; Su et al. (1999) J. Immunol. 162:5924-5930). Leucine zippers comprise a motif comprising parallel α-helical coiled coils as exemplified by those found in transcription factors GCN4 (RMKQLEDKVEELLSKNYHLENECARLKKLVGER) (SEQ ID NO:316), Fos (LTDTLQAETDQLEDKKSALQTEIANLLKEKEKLEFILAA) (SEQ ID NO:317), and Jun (RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMN) (SEQ ID NO:318). The motif is represented by the occurrence of leucines in every seventh position and hydrophobic and branched amino acids occupying position 1 over four or five heptad repeats. These motifs act as molecular clamps and facilitate homo- and hetero- dimer formation of proteins. Homodimerization or higher order self-oligomerization of a protein is also accomplished using other zipper motifs (e.g., Zhang et al. (1999) Current Biol. 9:417-420) or mutated peptide sequences derived from transcription factors, GCN4, Fos, or Jun.

10. Delete the paragraph beginning at page 238, line 15, and substitute therefor the following paragraph:

C10

Soluble monomeric NCSM molecules are modified with leucine zippers using standard molecular biology methods utilizing either natural (genomic eukaryotic/prokaryotic DNA or plasmid/viral DNA) or synthetic DNA encoding known or newly discovered oligomerization motifs. These sequence motifs are engineered as molecular zipper cassettes containing unique restriction enzyme sites to make in-frame fusion with either the N-terminus or C-terminus of a soluble monomeric NCSM. For example, a DNA sequence encoding the leucine zipper comprising the restriction site Not I at the 5' end and restriction site Apa I at the 3' end is used to facilitate cloning into a vector backbone, such as, e.g., pCDNA3.1 (GCGGCCGCa<GCN4>TAGGGGCCC (SEQ ID NO:319) ; GCN4 nucleotide sequence can be codon optimized to improve translation in a particular cell of interest, such as a human cell). This allows for easy shuttling of DNA fragments encoding soluble monomeric NCSM-ECD polypeptides or monomeric NCSM-ECD Ig-fusion polypeptides to oligomerization expression vectors. These expression vectors can also include an E-epitope and hexa-His tag for diagnostic and purification of soluble proteins.

11. Delete the paragraph beginning at page 240, line 6, and substitute therefor the following paragraph:

C11

The ColE1 origin was isolated by polymerase chain reaction (PCR) methods known in the art from vector pUC19 (New England Biolabs Inc.). To link the ColE1 origin to the Kana^r gene, NgoMIV (or "NgoMI") and DraIII recognition sequences where added to the 5' and 3' PCR primers, respectively. NgoMIV and DraIII are unique cloning sites in the vector. For subsequent cloning of the mammalian transcription unit the 5'forward primer contains the additional restriction site NheI downstream of the NgoMIV site and the 3'reverse primer additional EcoRV and BsrGI cloning sites upstream of the DraIII site. All primers contain additional 6 – 8 base pairs overhang for optimal restriction digest. The sequence for the 5' forward primer is: acacatagcgccggcgctagctgagcaaaaggccagcaaaaggcca (SEQ ID NO:302). The sequence for the 3' reverse primer is: aactctgtgagacaacagtcataaatgtacagatatcagaccaagtttactcatatatac (SEQ ID NO:303). The PCR reactions are usually performed with proof-reading polymerases, such as Tth (PE Applied Biosystems), Pfu, PfuTurbo and Herculase (Stratagene), or Pwo (Roche), according to the

manufacturer's recommendations. A typical PCR reaction for Herculase polymerase contains 1 µl template plasmid DNA (1-10 ng/ µl), 5 µl 10x buffer, 1 µl dNTPs (deoxynucleotide triphosphate) at 10 mM each, 1 µl forward primer (20 µM), 1 µl reverse primer (20 µM), 40 µl deionized, sterile water and 0.5 µl Herculase polymerase in a 50 µl reaction. The PCR reaction is performed at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds per cycle, for a total of 25 cycles. The PCR products were purified with phenol/chloroform using Phase lock Gel™Tube (Eppendorf) followed by standard ethanol precipitation. The purified PCR products were digested with the restriction enzymes NgoMIV and DraIII according to the manufacturer's recommendations (New England Biolabs, Inc.) and gel purified using the QiaExII gel extraction kit (Qiagen) according to the manufacturer's instructions.

12. Delete the paragraph beginning at page 241, line 1, and substitute therefor the following paragraph:

The 5' PCR primers contain the DraIII cloning site and an additional single restriction site, AscI, downstream of it. The 3' PCR primers contain the NgoMIV cloning site. The 5'forward primer sequence is: ggcttctcacagagtggcgcgccgtgtctcaaaatctct (SEQ ID NO:304). The sequence for the 3' reverse primer is: ttgctcagctagcgccggcgccgtcccgtcaagtcagcgt (SEQ ID NO:305). The PCR reactions, product purification and digest with DraIII and NgoMIV were performed as described above. About 20 ng of each of the two PCR products were ligated in a 20 µl reaction, containing 2 µl 10x buffer and 1U ligase (Roche). Amplification in E. coli was performed using standard procedures as described in Sambrook, *supra*. Plasmids were purified with the QiaPrep-spin Miniprep kit (Qiagen) following the manufacturer's instructions and digested with BsrG1 and DraIII for subsequent ligation of the mammalian transcription unit (promoter and polyA).

13. Delete the paragraph beginning at page 242, line 5, and substitute therefor the following paragraph:

The polyadenylation signal from the bovine growth hormone (BGH) gene was used in this example. Other poly A signals, which work well in mammalian cells, include, e.g.,

poly A signal sequences from, e.g., SV40, Herpes simplex Tk, and rabbit beta globin, and the like, and others known to those of skill in the art. The BGH poly A was isolated from the pCDNA3.1 vector (Invitrogen) using commonly known PCR methods. The 5' PCR forward primer contained additional 14 bp sequence comprising recognition sites for the restriction enzymes PmeI and BglII, which form part of the poly linker. The 3' reverse primer contains the restriction site DraIII for cloning to the minimal plasmid Col/Kana. The 5'forward primer sequence is: agatctgtttaaaccgctgatcagcctcgactgtgccttc (SEQ ID NO:306). The 3'reverse primer sequence is: acctctaaccactctgtgagaagccatagagcccaccgca (SEQ ID NO:307). The resulting PCR product was diluted 1:100, and 1 µl was used as a template for a second PCR reaction with the same 3'reverse primer and a new 5' forward primer. This primer was overlapping the 5' end of the template by 20 bp and contained another 40 bp 5', containing BamHI, KpnI, XbaI, EcoRI and NotI recognition sequences to form the rest of the polylinker. The sequence of the 5'extension primer is: ggatccggtacctctagagaattcggcggccgcagatctgtttaaaccgctga (SEQ ID NO:308). An alternative PCR product was generated with different 5'forward PCR primers to generate a vector with a modified polylinker, designated pMaxVax10.1mp (Fig. 21 with modified polylinker as described above). The orientation of the restriction sites in this polylinker is 5'-3': BamHI, XbaI, KpnI, EcoRI, NotI, BglII, and PmeI. The polylinker sequence is: ggatccactcatctagaacaatggtaccaatacgaattcggcggccgcagatctgtttaaacc (SEQ ID NO:309). The PCR products were digested with BamHI and DraIII and gel purified.

14. Delete the paragraph beginning at page 244, line 29, and ending at page 245, line 13, and substitute therefor the following paragraph:

The CD28BP gene is inserted into the polylinker of a pMaxVax vector as described above, forming the first expression unit. The nucleic acid sequence of the cancer antigen, here the polynucleotide encoding the extracellular domain of EpCAM/KSA (or mutant or variant thereof), is linked to a second mammalian expression promoter (exemplary promoters include those set forth in this Example above and elsewhere) and a second poly A signal (exemplary signals include those set forth in this Example above and elsewhere) to form the second expression unit. In this Example, a synthetic poly A (SPA) sequence was made and used.

However, one of skill in the art would understand that other poly A sequences (e.g., bovine growth hormone (BGH) poly A or SV40 poly A sequence) can also be used. The synthetic poly A was derived from a sequence for the rabbit β-globin poly A (Gen&Dev. 3:1019-1025 (1989). The sequence fragment was generated by annealing two oligonucleotides, which contained the respective cloning sites in the 5' and 3' sequences. The upper strand sequence is: 5'-GATCTGTTTAAACTCTGGCTAATAAAAGATCAGAGCTCTAGACATCTGTGTGTTGGTTTTTTGTGTGTCTCACTCACAGA-3' (SEQ ID NO:313), and the sequence of the lower oligonucleotide strand is: 5'-TGAGTGAGACACACAAAAAACCAACACACAGATGTCTAGAGCTCTGATCTTTTATTAGCCAGAGTTTAAACA-3" (SEQ ID NO:314).

15. Delete the paragraph beginning at page 245, line 14, and substitute therefor the following paragraph:

The second expression unit can be cloned into 3 different sites in the construct pMaxVax-CD28BP, both in forward or reverse orientation: (i) downstream of the first expression unit (e.g., CMV promoter-CD28BP-SPA polyA, CMVpromoter-CD28BP-BGH polyA, or CMVpromoter-CD28BP-SV40 polyA) using the single cloning sites DraIII and AscI in pMaxVax10.1; (ii) between the ColE1 and Kana^r gene using the single restriction sites NgoMI and NheI; (iii) between the Kana^r gene and the CMV promoter into the single EcoRV and BsrGI restriction sites (see vector description above in this Example). Independent of the location of the second expression unit it is advisable to add a terminator sequence downstream of the first expression unit. A consensus terminator sequence 5'-ATCAAAA/TTAGGAAGA3' (SEQ ID NO:310) is described in Ming-Chei Maa et al. (1990) JBC 256 (21):12513-12519. In the construct pMaxVax,CD28BP the sequence can be placed into the single DraIII site downstream of the poly A sequence (e.g., synthetic poly A or SPABGH poly A sequence) (see Figure 22B).

16. Delete the paragraph beginning at page 123, line 7, and substitute therefor the following paragraph: